The Art of Silence

The transition from biology to technology is never as easy as it sounds, and taking technology out of the hands of artisans for widespread use adds another level of difficulty. Natasha Caplen, *Ph.D.*, Head of the Gene Silencing Section in *CCR's* Genetics Branch, *first recognized the gap between understanding in principle and implementing in practice as a* Postdoctoral Fellow *working on gene therapy for cystic fibrosis. One of the first disease genes cloned, cystic fibrosis genes still elude therapeutic attempts to replace them with functional copies.* While working at the National Human Genome Research Institute *on gene delivery methods,* Caplen *was first exposed to* RNA interference—*RNAi—and was among the first to generate the phenomenon in mammalian cells.* Less than 10 *years later, RNAi is a much valued technique for studying gene function, but its nuanced execution still demands an artisanal approach.*

"In March 2001, when I first observed the effect of RNAi in mammalian cells on the 10th floor of Building 10, I ran around the lab saying 'They aren't green any more!'" Caplen often tells this story in seminars of how she first silenced a reporter gene that encoded green fluorescence protein (GFP). "For 18 months, I looked at very dead mammalian cells because if you put double-stranded RNA into cells, they trigger immune responses and die. But I thought there had to be a way."

RNAi is a gene silencing mechanism that was first identified in nematode worms where it operates to modulate gene expression. Double-stranded RNA (dsRNA) is processed in cells to form small interfering RNAs (siRNAs) that contain 21-23 nucleotides. These siRNAs direct the breakdown of gene transcripts that contain complementary sequences and thereby silence gene expression. Although dsRNA provokes mammalian immune responses, Caplen and her colleagues discovered that siRNAs can be used directly as an experimental tool in mammalian cells.

"I don't think anyone at the time could have really realized what we were letting loose into the world," said Caplen. "We've all been stunned by how RNAi is being adapted—the imaginative ways people have used this basic mechanism and the implications that it has."

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In fact, three years later, in 2004, Caplen was recruited to CCR because it had rapidly become clear that no large cancer research center could be without expertise in RNAi. Since then, she has embarked on several collaborations with investigators studying diverse models of cancer who want to use RNAi technologies.

Any Individual Gene

Thomas Ried, M.D., Head of the Cancer Genomics Section, has been studying colorectal cancer (CRC) for several years with the goal of understanding changes that happen early in the transformation from normal epithelium through dysplasia or polyps into full blown carcinoma. "Our interest is to identify the dynamics of genomic and transcriptomic changes that occur in early tumorigenesis and are responsible for the change from a benign dysplasia to a carcinoma."

(*Photo: R. Baer*)

Natasha Caplen, Ph.D.

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Over the course of their studies, Ried and his colleagues have uncovered several genes that are consistently upregulated in CRC tumors and cell lines. "It is very reasonable to believe that they are necessary for growth and viability of these cells," said Ried. "And of course, if you identify genes that are exclusively expressed in cancer but not normal colon, you can assume that they are viable targets." But, reasonable hypotheses and assumptions are not proof. RNAi seemed like one obvious strategy to silence these genes and establish their roles in supporting CRC.

"We have done a lot of thinking as to what it means to silence a single cancer gene," said Caplen. When an investigator comes to her with a project, she wants to be sure that she can match the best technologies with the best assays and analysis tools. In terms of RNAi resources, this means analyzing the architecture of the transcripts produced by any one gene as well as the potential for unwanted interactions with other nucleic acid elements. John Weinstein, M.D., Ph.D., former Head of the Genomics and Bioinformatics Group at CCR, and Mike Ryan, Ph.D., worked with Caplen to create Web sites that help determine how well a siRNA sequence will line up with and thereby silence different gene isoforms and, more recently, identify potential off-target interactions of siRNAs. "It's those kinds of tricks that can help you identify sources of any inconsistency in your data."

Although there are newer and fancier methods for building RNA molecules to silence genes that the lab keeps abreast of, synthetic siRNAs serve Caplen and her colleagues well for most applications. "Building experience and tools to interpret the data and using the right assay has been more of our focus than building more RNAi resources per se," said Caplen.

Because Caplen works predominantly in cell lines that model cancers, she also wants to make sure that the model is as faithful a reflection of the tumor biology as possible. "These experiments can get very big very quickly, so you really want to know that what you have is modeling the question well," explained Caplen. She often asks her collaborators for additional characterization of the cell lines they are using.

"The fidelity of cancer cell line models has been a concern for many years," said Ried. "However, we have found that both the genomic aberration profile, which is very specific for certain tumors, and the transcriptome profile of our cell lines match the tumors very well. So my confidence has actually grown for using tumor cell lines."

An exciting outcome of silencing experiments is to look at several snapshots in time of changes in gene expression across the entire

molecular network as a result of a single inactivation event induced by RNAi. "If you perturb gene X and look at a whole transcriptome level at what happens 10 hours later, 24 hours later, etc.,…now you can start to use systems biology to model the pathways that are really being affected over time in a way that needs no assumptions about the role of the gene you silenced.

"That gives you a lot of information," said Ried. "The challenge will be doing the right bioinformatic analysis—nobody knows exactly what that is. But this approach should allow us to identify the functional space in which a single gene operates. The gene expression signature of the knockdown also might tell us whether inhibiting a combination of genes might give an additive effect rather than just targeting the same pathway."

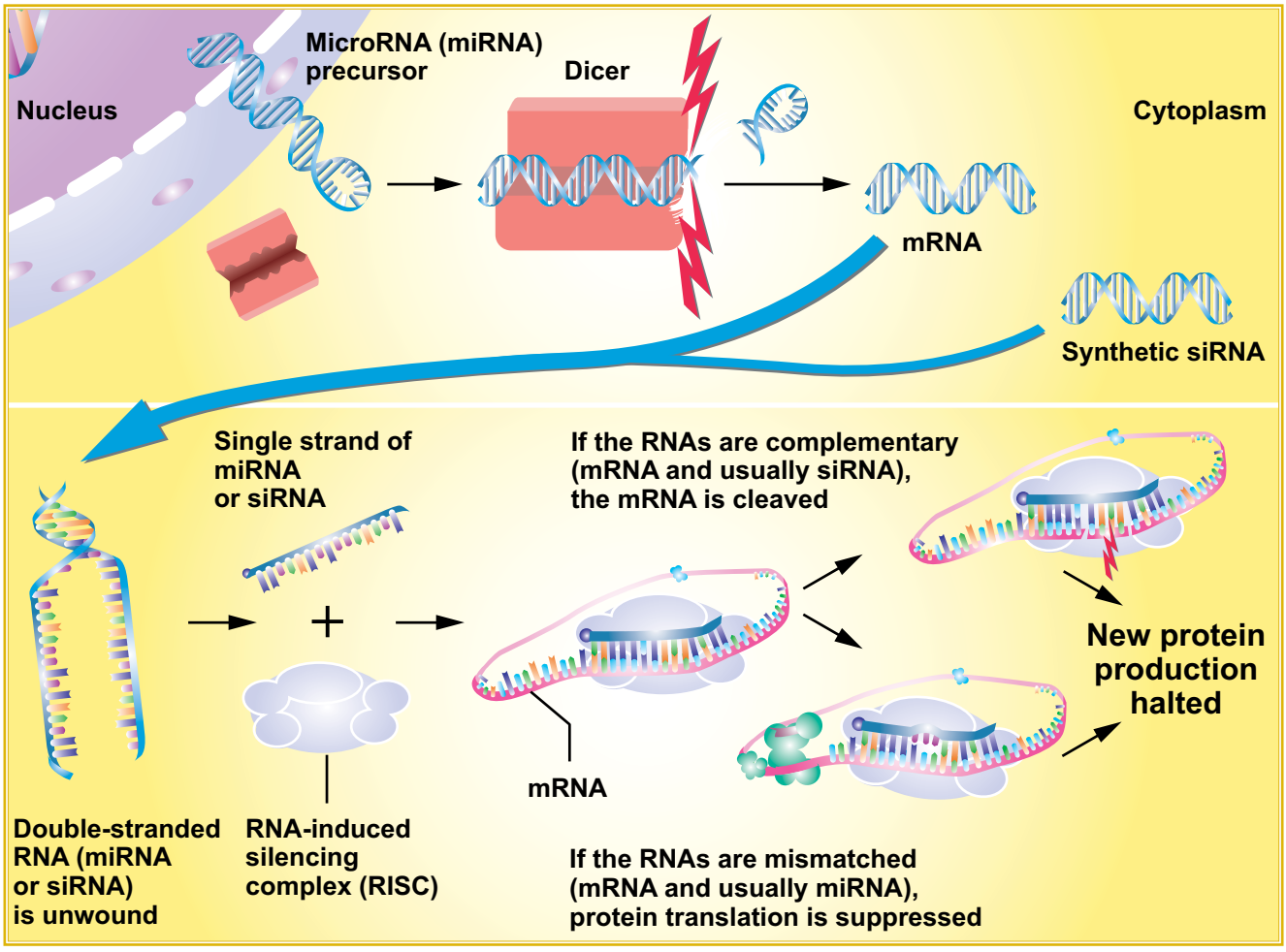
Many, Many Genes

Anita Tandle, Ph.D., Staff Scientist in the Radiation Oncology Branch under the direction of Kevin Camphausen, M.D., is also trying to find gene targets in cancer cells, but she is looking for genes that might sensitize glioblastoma stem cells to the effects of radiation.

"Most of these gliomas are managed by surgery and radiation, but the median survival can be counted in months. We want to see if we can identify proteins or genes that make these tumors more sensitive to radiation."

The Camphausen group, in collaboration with Philip Tofilon, Ph.D., at the Moffitt Cancer Center, has developed a glioblastoma stem cell line. "The proportion of cancer stem cells is very small in a tumor, but they are responsible for resistance that develops to radiation and chemotherapy," explained Tandle. She and her colleagues want to identify genes that confer such unique viability on these specialized cells and ultimately discover ways in which to make them vulnerable to radiation.

"We have relatively large libraries of siRNAs, in which you run your assay in 384 well plates just like drug screens," explained Caplen. "We can run one screen a week in which we ask a specific question." For example, which genes affect the growth of a glioblastoma stem cell? To answer this question, the researchers use four siRNAs per gene that they silence and the readout is either cell



(Image: J. Kelly)

To induce gene-specific inhibition of protein production, synthetic small interfering RNAs (siRNAs) exploit aspects of the naturally occurring RNAi mechanism that includes the control of gene expression by microRNAs (miRNAs).

(Photo: R. Baer)

Kristen Gehlhaus, M.H.S.

proliferation or cell death. Kristen Gehlhaus, M.H.S., a Biologist in Caplen's lab, has been handling the RNAi screen for this project.

Unlike other cancer cell lines, glioblastoma stem cells grow as neurospheres and are actually quite tricky to work with. "They don't grow as monolayers, plus they are much slower growing than other tumor cell lines," explained Tandle.

"This is a great example of true team science," noted Caplen. "We bring the RNAi expertise and each collaborator brings their particular cancer biology expertise."

Caplen's group has used the same siRNA libraries to look at growth of breast cancer cell lines for another collaboration. Each collaborator has been focused on his own model of cancer, but Caplen sees a third angle of investigation that her lab will pursue independently—the differences in screening data across cell lines. "We're going to run with those differences," said Caplen. "In the same experiment, there is the potential to look at basic mechanisms for how a gene promotes the tumorigenic process as well as looking at pathways that you can target therapeutically. It is a lot of work following up on either of those results, but at least you have a rich source of leads."

Drugs, Too

Ultimately, Tandle and Gehlhaus will run their screens to look for genes that confer survival on glioblastoma stem cells after they have been irradiated. Caplen's group has been using RNAi screens with increasing frequency to study the genes that modulate the response to specific interventions. Most often, these interventions are drugs.

Stanley Lipkowitz, Ph.D., Senior Investigator in the Laboratory of Cellular and Molecular Biology, is interested in a family of receptors that trigger cell death: the tumor necrosis factor (TNF) family. Tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) is a member of that family that is currently in clinical trials as a chemotherapeutic agent. It turns out that most breast cancers are not responsive to TRAIL, but Lipkowitz and his colleagues have shown that a subset of breast cancer cells—the so-called triple negative tumors—are sensitive to TRAIL. "We don't know why, despite having studied several candidate genes," said Lipkowitz. So, they decided to try an RNAi screen, looking at roughly 1000 genes.

In addition to RNAi they gave cells TRAIL at IC_{50}, i.e., the concentration at which exactly half of the cells would die. The screen will give them information about genes that either makes these cells more resistant or more sensitive to TRAIL. The screen is now complete and for Lipkowitz, "the hard part begins."

"The drug studies have worked out very well," said Caplen. "We have had one published and a number of others that are coming out." In collaboration with Yves Pommier, M.D., Ph.D., Caplen's team has identified genes that affect the mechanism of action for camptothecins, a venerable class of anti-cancer drugs. And they have just begun a collaboration with Beverly Mock, Ph.D., trying to study how two different pathway inhibitors interact in cancer cells to produce synergistic effects on cell death.

Finding the Balance

RNAi, of course, is much more than just a tool for manipulating experiments. It is also a fascinating field of biology that has been transformed in the last decade. RNA molecules are involved in directing cellular activity at a number of levels and, in cancer research, the hottest new members are microRNAs (miRNA), very short molecules that modulate whole networks of genes. "The great unknown is how to find efficient and sustaining ways of linking miRNAs with their dominant targets in specific settings." When they are not honing their RNAi technologies, Caplen and her team are busy investigating the secrets of RNAi biology. The challenge for Caplen is in creating the right mix of projects in an environment whose structure is different from a traditional laboratory. During

a recent site visit, one outside examiner said of her laboratory, "It would be almost impossible to replicate anywhere else."

Caplen is justifiably proud of her unique niche and the important role it plays in developing not just the biology and technology of small RNA molecules, but also the next generation of team scientists.

"The first thing I tell every postdoc joining the lab is to make sure they are aware that this is a team science environment," said Caplen. "There is a continual balance between their own projects and working with other groups."

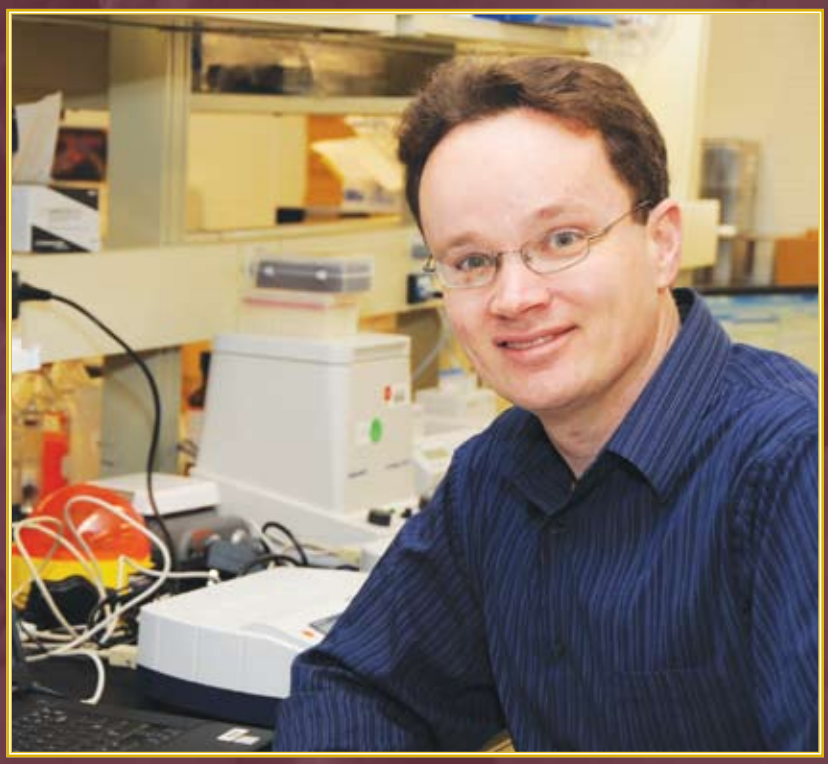
Scott Martin, Ph.D., came to the Caplen laboratory after his doctoral work developing small molecule probes for biological systems. "I became more interested in biology and decided to immerse myself in cancer biology." He found the Caplen laboratory an ideal interface between his prior training and newfound interests. "RNAi made sense as an extension of my work developing reagents to alter molecular activity in cells."

Martin found a great deal of freedom in the lab. "You can pursue whatever you want as long as it is RNAi related. I was interested in drug sensitization; other people were off investigating miRNA biology." Although he engaged in many rewarding projects during his four years in the lab, he is most proud of discovering genes that sensitize breast cancer cells to camptothecins.

Martin has recently joined the NIH Chemical Genomics Center (NCGC) to lead a new RNAi initiative (see "NIH Chemical Genomics Center Takes in RNAi"). "So, this is sort of full circle for me. I thought I was a jack of all trades kind of person and it would be difficult to find a perfect fit, but there I found it."

To learn more about Dr. Caplen's research, please visit her CCR Web site at ***http://ccr.cancer.gov/staff/staff.asp?Name=caplen****.*

NIH Chemical Genomics Center Takes in RNAi



(*Photo: R. Baer*)

Former CCR Fellow Scott Martin, Ph.D., now leads the RNAi screening initiative at NCGC.

A new RNAi screening facility based in the NIH Chemical Genomics Center will serve intramural investigators across the whole of NIH. "The goal is to provide access to genome-wide RNAi screening, primarily in human cells," explained Scott Martin, Ph.D., who is leading the project.

"We envision this as being a highly collaborative type of process, starting with discussions with investigators to help them get an assay that addresses the biology that they are interested in," said Martin. The project would then have to be approved by a trans-NIH committee, headed by Natasha Caplen, Ph.D.

Once approved, the Center will actually perform the screening and provide real-time feedback. At the end of the project, the investigator will be provided with a list of hits and all the data associated with activities in the screen.

So far, the infrastructure is in place and pilot studies are ongoing. "It was a huge advantage to build this on top of the physical and informatics infrastructure that the NCGC has in place to do large chemical compound screens," noted Martin. They hope to be fully operational by 2011.